# NOTE ON THE BACTERIOTOXIC ACTION OF WATER.

By R. Greig-Smith, D.Sc., Macleay Bacteriologist to the Society.

The presence of bodies, toxic to bacteria, have been known to occur in water for some time, just as their existence in soils has been suggested. Sidney Martin,* in 1900, showed that when typhoid bacteria are added to a well moistened, cultivated soil, they rapidly die out, and are not usually obtained two days afterwards. The same occurs when the typhoid bacillus is added to a culture of a soil-microbe in bouillon; it rapidly disappears. Sidney Martin explained this phenomenon by the bacteria in general being destroyed by the products of putrefaction, which exist in most cultivated soils. Referring to the growth of the typhoid bacillus, he said that it cannot grow except in the presence of organic matter containing nitrogen, and, on this account, it grows for only a short time in sterilised distilled or tap-water. That there is something more than the absence of organic matter to explain its disappearance in sterilised water, may be inferred from his experiment with the bouillon-culture of the soil-bacillus.

Typhoid bacteria rapidly disappear in sewage which contains a certain amount of nitrogenous, organic matter. For example, Houston† added 205 per 0·01 c.c. of sewage, and as he could recover only 20, he concluded that the remainder had been destroyed.

With regard to the growth of bacteria in waters, Miguel,‡ in 1891, noted that a rapid, but transitory, increase occurred in spring waters, while, in impure waters, the increase was slow but

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* Loc. Govt. Rept. 1900. Suppl., p.487.

† Metrop. Water Board, Ninth Research Rept., through Journ. Soc. Chem. Ind. 1913, 764.

‡ Frankland, Micro-organisms in Water, London, 1894.

persistent. He found that, after a water had supported the multiplication of a particular species of micro-organism, the latter, on being reintroduced into the same water, will not only not again multiply, but, in many cases, will actually suffer rapid destruction. He compared this immunity of the water to the generation by the bacteria of soluble and toxic products, which inhibit their further growth and multiplication. These soluble products can be concentrated by evaporation at a low temperature, but are destroyed by boiling.

The effect of boiling is noteworthy, for while Martin says that typhoid bacteria soon perish in sterilised (*i.e.*, boiled) water, Meade Bolton found that water-bacteria increase enormously when introduced into sterilised water kept at 22°. That this depends upon the individual species of bacteria, has been shown by Rosenberg, who introduced a series of water-organisms into sterilised distilled water, and found that, while the majority of the individual varieties multiplied quickly, three of the species rapidly died out.

Having shown that bacteriotoxins can be demonstrated in soils, that they are produced there and are leached out by rain, it appeared to me to be a natural corollary that they should be found in drainage-waters. Furthermore, they ought to occur in natural waters in which bacteria are growing, although the quantity will, in all probability, be small and somewhat proportional to the number of bacteria which are present. In pursuance of the idea underlying my work upon the bacteriotoxins of soils, a few experiments were made with Sydney tap-water. This is an unfiltered water; the only purification to which it is subjected, consists of being strained through a series of sieves of a fine mesh at the city-reservoirs. It contains few bacteria; for example, on September 22nd, 1913, the date of the first experiment, at a temperature of 16°, it was found to contain 140 bacteria per c.c. when seeded into Lipman's synthetic agar.

The experimental method consisted in filtering the water through porcelain filters (Chamberland F) and adding one c.c. of a suspension of *Bac. prodigiosus* to 10 c.c. of the water, unboiled and after having been boiled for 15 and 60 minutes under a

reflux condenser. The suspensions were incubated overnight, usually for 20 hours, at 22°, and were then counted by the plate-method. The experimental error was minimised by making four tests of each, and making five plate-counts of each test.

Experiment i.

| Date. | Temperature of water. | 100 cells of *Bac. prodigiosus* became | | |
|---|---|---|---|---|
| | | Unboiled. | Boiled | |
| | | | 15 minutes. | 60 minutes. |
| Sept. 22nd, 1913 ..... | 16° | 136 | 182 | 259 |
| Oct. 13th, 1913..... | 19° | 246 | 191 | 73 |
| Oct. 15th, 1913..... | 20° | 155 | 50 | 25 |
| Oct. 23rd, 1913 ..... | 20° | 230 | 162 | 66 |
| Oct. 29th, 1913..... | 19° | 480 | 164 | 112 |
| June 10th, 1914 ..... | 15° | 134 | 156 | 71 |
| June 17th, 1914 ..... | 14° | 217 | 202 | 130 |
| July 1st, 1914 ..... | 14° | 98 | 108 | 119 |
| | Average .. | 212 | 152 | 107 |

The following numbers for raw filtered water are taken from the control-tests of other experiments.

| | 100 cells of *Bac. prodigiosus* became |
|---|---|
| May 25th, 1914 ..... ..... | 74 |
| June 4th, 1914 ..... | 49 |
| June 12th, 1914 ..... | 71 |
| June 18th, 1914 ..... | 136 |
| June 30th, 1914 ..... | 86 |
| July 2nd, 1914 ..... | 112 |

The action of unboiled and boiled filtered tap-water upon *Bac. prodigiosus* is irregular, possibly on account of the water itself varying, but the general tendency is for the boiled and cooled water to retard the growth of the bacteria suspended in it.

A sewage-effluent was obtained through the kindness of Dr. Stokes, Medical Officer to the Metropolitan Water and Sewerage Board, and tested in a similar manner. The sample was received on the 7th of October, 1913, when it was examined. It was then stored in a laboratory cupboard for a fortnight and again tested. It contained 90,000 bacteria per c.c. on October 21st. The temperature during storage was 22°.

Experiment iii.

| Date. | 100 cells of *Bac. prodigiosus* became | | |
|---|---|---|---|
| | Unboiled effluent | Boiled effluent. | |
| | | 15 minutes. | 60 minutes. |
| October 7th | 400 | 86 | 31 |
| October 21st | 463 | 41 | 35 |
| Average | 431 | 63 | 33 |

The unboiled, filtered effluent was nutritive to *Bac. prodigiosus*, and the boiled effluent was decidedly toxic.

While *Bac. prodigiosus* has been taken as an organism capable of indicating the existence or otherwise of the toxic or nutritive effect, it appeared advisable to test *Bac. typhi*. Accordingly, cultures of two strains were obtained from Dr. Cleland, of the Board of Health, and the previous experiments with tap-water were repeated.

Experiment iv.

| Date. | Temperature of water. | Strain. | 1,000 cells *Bac. typhi* became | | |
|---|---|---|---|---|---|
| | | | Unboiled. | Boiled. | |
| | | | | 15 minutes | 60 minutes. |
| Nov. 5th, 1913 | 20° | "976" | 460 | 2 | 0 |
| Nov. 11th, 1913 | 20° | "L.I.P.M." | 452 | 11 | 6 |
| June 2nd, 1914 | 14° | "L.I.P.M." | 557 | 2 | 0 |

*Bac. typhi* is very susceptible to the action of tap-water, which is toxic to it; and, as in the previous experiments, boiling increased the toxicity.

One of the quadruplicate tests of the last experiment was incubated for a further period of 24 hours to determine if the toxic action continued.

EXPERIMENT V.

| | 1,000 cells of *Bac. typhi* at 22° became | |
|---|---|---|
| | in 20 hours | in 44 hours. |
| Water unheated..... | 431 | 192 |
| Water boiled 15 minutes .. | 6 | 6 |
| Water boiled 60 minutes ..... | 2 | 2 |

The toxic action continued in the unheated water, while, in the boiled water, the bacteria persisted unaltered.

The reduction in the numbers of bacteria put into ordinary filtered water points to some injurious factor, and the increased reduction in the same water, when boiled, seems to indicate that the effect is not caused by an absence of organic matter. If, however, we ignore the action of the boiled water, a possible explanation for the destructive effect of raw water might be found in the shock given the bacteria by the transference from a saline media, such as ordinary nutrient agar, to water virtually devoid of saline matter. To test this possibility, the bacteria were grown in sodium chloride-free nutrient agar for several generations, and the experiment again made.

EXPERIMENT VI.

| Date. | Temperature of water. | Strain. | 1,000 cells *Bac. typhi* became | | |
|---|---|---|---|---|---|
| | | | Unboiled. | Boiled. | |
| | | | | 15 minutes. | 60 minutes. |
| Nov. 26th, 1913 | 22° | "L.I.P.M." | 562 | 10 | 3 |

The experiment shows that the reason for the destruction of the bacteria is not to be found in the lessened saline content of the media. We are justified, therefore, in concluding that ordinary tap-water contains substances of the nature of bacterio-toxins, the toxicity of which is increased by boiling.

## NOTE ON THE DESTRUCTION OF PARAFFIN BY *BAC. PRODIGIOSUS* AND SOIL-ORGANISMS.

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In an earlier research, in which dried blood had been saturated with paraffin or vaseline, and subsequently fermented, it was found that the treatment did not prevent the blood from being attacked by soil-bacteria, or by a pure culture of *Bac. prodigiosus*. This was not expected, as it was believed that the inert hydrocarbon would offer a barrier to the attack of the bacterial enzymes. The failure was possibly due to the organic matter swelling upon being moistened, and thus breaking the paraffin-covering, but, that some other action was possible, was suggested by the behaviour of the fermented blood when shaken up with water, as compared with the fermented controls.

It has recently been shown that certain bacteria and moulds are capable of utilising both solid and liquid hydrocarbons,* and an experiment was undertaken to see if there might be a similar utilisation by *Bac. prodigiosus*.

Sifted dried blood was saturated with paraffin, and the excess removed as completely as possible. The paraffined blood was again sifted and thoroughly mixed. Two-gram portions were mixed with 50 grams of sand and moistened with a suspension of *Bac. prodigiosus*. The tests were kept at the temperature of the laboratory for varying periods, then dried and extracted with ether.

| | Paraffin recovered. |
|---|---|
| Control ... ... ... ... | 0·169 grams. |
| | 0·164 ,, |
| | 0·164 ,, |
| Fermented for 10 days ... ... | 0·155 ,, |
| | 0·157 ,, |
| Fermented for 17 days . . ... .. | 0·155 ,, |
| Fermented for 57 days ... ... ... | 0·157 ,, |

* Rahn, Centrlbl. f. Bakt. 2te Abt. 16, 362; and Söhngen, *ibid.* 37, 595.

The loss of paraffin is approximately 5 %. The melting points of the recovered paraffin from the control and 10 days' tests were determined, and both were found to be the same, viz., 45°.

Powdered casein, the granules of which passed through a No.30 sieve, was used in the next experiment. About ten grams of paraffin were added to 130 grams of casein, and the mixture was heated at 100° and stirred until it was considered that the hydrocarbon had been evenly distributed. It was then cooled, powdered, and sifted. Fifteen-gram portions were weighed out, placed in beakers, mixed with four grams of kieselguhr, and moistened with 20 c.c. of water containing a suspension of *Bac. prodigiosus*.

| | Paraffin recovered. |
|---|---|
| Control ... .. ... ... ... | 1·37 grams.<br>1·35 ,, |
| Fermented 11 days at 37°... .. ... | 1·05 ,, |
| Fermented 11 days at 22°... ... ... | 1·20 ,, |
| Fermented 17 days at 22°... .. ... | 1·17 ,, |
| Fermented 24 days at 22°... ... ... | 1·07 ,, |

The loss of paraffin ranges from 11 % to 22 %.

During the direct estimation of the total paraffin, several observations showed that the method of recovery was capable of improvement. When the cultures were spread out to dry, previous to the ethereal extraction, a strong odour of ammonia was given off, and this was followed by a pungent gas, sometimes resembling formaldehyde, at others acrolein. This led to the 17 and 24 days' tests being mixed with sodium carbonate and powdered lime, respectively, before drying. But even this did not prevent the presence of volatile acids in the recovered paraffin. A trace of lecithin was also present. A method was accordingly employed in the later experiments that eliminated these objectionable substances.

The method simply consists in digesting the paraffin recovered by the Soxhlet apparatus, with 5 c.c. of 10 % sodium hydrate, transferring to a 50 c.c. graduated Wehrner-Schmit tube, allow

ing the alkaline liquid to measure about 20 c.c., adding ether to the 49 c.c. mark, and, in the casein tests, adding 1 c.c. of rectified spirit. After a vigorous shaking, the ethereal layer is read off, and 10c.c. portions pipetted out into flat-bottomed, metal evaporating dishes. The ether is allowed to evaporate at 30°, and then dried for two hours in the water-oven. The procedure is similar to that employed in the estimation of fat in milk by the Wehrner-Schmit process.

Fifteen grams of paraffined casein were mixed with one gram of kieselguhr and one gram of calcium carbonate, placed in two-ounce bottles, and, after sterilisation in the autoclave, treated with 5 c.c. of a suspension of *Bac. prodigiosus* or of soil-organisms (5 grams of soil to 100 c.c. of water). The bottles were incubated at 28°.

| | Paraffin recovered. |
|---|---|
| Control ... ... ... .. ... ... | 1·21 grams.<br>1·22 ,, |
| Fermented with soil-organisms, 15 days ... | 1·10 ,,<br>1·07 ,, |
| Fermented with *Bac. prodigiosus*, 25 days ... | 1·18 ,,<br>1·16 ,, |

The average loss for the soil-organisms is 11·1 %, and for *Bac. prodigiosus* 3·7 %.

Finally, an experiment was made in which the bulky, organic, nitrogenous matter was dispensed with. Kieselguhr was treated with paraffin, ground and sifted. Four grams were mixed with 20 grams of sand and one gram of calcium carbonate, and the bottles containing the mixture were sterilised in the autoclave. Ten c.c. of water containing 0·1 % dipotassium phosphate, 0·05 % magnesium sulphate, 0·05 % sodium chloride, and either 1 % peptone, 0·5 % asparagin, or 0·5 % ammonium sulphate were added. The bottles were then steamed for an hour, and infected with 2 c.c. of a suspension of *Bac. prodigiosus*, containing 12 million cells, or 2 c.c. of a suspension of soil-organisms (10 grams

of soil to 100 c.c. of water). The bottles were kept at 28°, and water was added occasionally to maintain the original weight.

| | Paraffin recovered. | |
|---|---|---|
| | *Bac. prodigiosus* | Soil-organisms. |
| Control ... ... ... ... ... | 0·92 grams. | 0·92 grams. |
| Peptone, 10 days .. ... ... | 0 92 ,, | 0 66 ,, |
| Asparagin, 11 days ... ... . | 0·90 ,, | 0·72 ,, |
| Ammonium sulphate, 21 days .. ... | 0·79 ,, | 0·66 ,, |
| Peptone, 25 days ... ... .. | 0·80 ,, | 0·52 ,, |
| Asparagin, 33 days ... ... .. | 0·79 ,, | 0·47 ,, |

There is a loss of 14% of paraffin occasioned by *Bac. prodigiosus*, and of 49% by the soil-organisms, in about a month at 28°. The decomposition appears to be uninfluenced by the nature of the nitrogenous matter.